THESIS ABSTRACT

Santosh Kumar Dasari
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In mammals, freshly ejaculated spermatozoa do not possess the ability to fertilize a mature oocyte. They acquire fertilization competence upon residing for a period of time in the female reproductive tract. The physiological changes that bring about these time-dependent changes in motility pattern and acquisition of fertilizing ability of spermatozoa are collectively referred to as capacitation, culminating in sperm hyperactivation. Capacitation-associated increase in sperm protein tyrosine phosphorylation (PYP), exhibited by mammalian sperm, is one of the major downstream events, regulating hyperactivated motility. However, it is still unclear which are the tyrosine kinases and phosphatases involved in modulating the capacitation-associated increase in global PYP. In order to determine this, our laboratory earlier showed the role of PYP in hamster sperm capacitation using a specific EGFR protein tyrosine kinase (PTK) inhibitor, tyrphostin A-47 (TP-47). Interestingly, inhibition of capacitation by 0.5 mM TP-47 was associated with induction of a slow circular motility pattern, accompanied by inhibition of PYP of certain proteins (Mr. 45,000-52,000), localized to the principle piece of the sperm flagellum. Two such proteins, hypo-tyrosine phosphorylated, were found to be tektin-2 and ODF-2, using 2D-PAGE followed by MS/MS analysis. Interestingly, a global phosphoproteome analysis of human spermatozoa showed that PYP changes are associated with capacitation and asthenospermic condition in infertile men is attributed to the failure of capacitation-associated increase in PYP. Such individuals exhibited impaired sperm motility. There is a need to understand the exact mechanism of phosphorylation of sperm flagellar proteins, which is necessary to assess sperm's ability to fertilize the mature oocyte. Therefore, the focus of the present work was to elucidate the role of receptor tyrosine kinases (RTKs) and the non-receptor tyrosine kinases (NRTKs) in mammalian (hamster) sperm capacitation. Recent studies have shown that apart from EGFR other RTKs like IGF1R, FGFR, VEGFR, MuSK, TrkA are expressed in mammalian spermatozoa and actively involved in sperm capacitation. However, there is very little information available in the context of sperm capacitation and associated PYP. Therefore, attempts were made to understand the role of various RTKs (IGF1R, FGFR and VEGFR) in hamster sperm capacitation and associated PYP. Initially, the role of IGF1R tyrosine kinase during sperm capacitation was studied. Immunolocalization of IGF1R in spermatozoa showed a strong signal in the sperm acrosome and the principal piece of the sperm flagellum. Inhibition of IGF1R kinase with an IGF1R-specific inhibitor TP-1-O-Me-AG538 (TP-538) showed inhibitory effect on sperm capacitation and the associated hyperactivation. But, inhibitors of FGFR and VEGFR tyrosine kinases did not show such an effect. Interestingly, inhibition of IGF1R by TP-538 was associated with inhibition of PYP of certain proteins (Mr. 45,000-120,000), localized to head, mid piece and principle piece regions of the sperm flagellum. Phosphoproteomic analysis using 2D-PAGE-western blot with anti-phosphotyrosine antibodies identified 17 differentially phosphorylated protein spots. Out of the 17 spots, 12 were identified by MALDI-MS/MS analysis. The proteins identified to be differentially phosphorylated, upon inhibition of IGF1R, were PDHE1, ODF-2, Tubulin β 2C chain, PDHE2 and ATP synthase β subunit.

The RTKs being present in the membrane level may not be directly involved in the phopshorylation of downstream target proteins associated with the mitochondrial membrane,

sperm axonemal structures and outer dense fibers. Therefore, the RTKs may interact directly or indirectly with the downstream NRTKs, which may be involved in the phosphorylation of target sperm proteins. Till date, six different families of NRTKs are shown to be expressed in mammalian spermatozoa. The major family of NRTKs involved in sperm function is the Src family of kinases. However, there is very little information available in the context of sperm capacitation and the associated PYP. Therefore, studies were carried out to understand the role of Src family of NRTKs in sperm capacitation and associated PYP. Presence of active Src signaling was observed by the immunolocalization of activated Src (pY416) in the acrosome, mid piece and the principal piece regions of the sperm flagellum. Inhibition of Src family of kinase with a specific Src family kinase inhibitor PP2, showed inhibition of sperm capacitation and the associated hyperactivation. Inhibition of Src family of kinases with PP2 was associated with decrease in PYP of several proteins (Mr. 45,000-120,000), localized mainly to the mid piece region, followed by the principle piece region of the sperm flagellum. Phosphoproteomic analysis using 2D-PAGE-western blot with anti-phosphotyrosine antibodies identified 38 differentially phosphorylated protein spots. Out of the 38 spots, 16 were identified by MALDI-MS/MS analysis and these corresponded to seven proteins which included PDHE1, ODF-2, Tubulin β 2C chain, Tektin-2, GAPDS, PDHE2 and ATP synthase β subunit.

Additionally, the biochemical and molecular characteristics of the identified proteins were also studied. Bioinformatic analysis predicted the presence of phosphorylation motifs for several kinases and interestingly, all the proteins identified had a Src kinase motif. Comparing the current observations and the previous work in the laboratory, two proteins ODF-2 and Tektin-2 were found to be regulated by EGFR, IGF1R and Src family of kinases. Therefore, characterization of the capacitation-associated tyrosine phoposphorylated proteins ODF-2 and Tektin-2 was performed. By employing PCR and Northern blotting techniques, the presence of the transcripts of both the proteins was shown. Additionally, the ontogeny of expression of ODF-2 and Tektin-2 in hamster testis development was studied and the results indicated that the expression of both the proteins started from week 3 onwards till week 8. To confirm the meiotic stage-associated expression of ODF-2 and Tektin-2, germ cells were sorted based on their DNA content. ODF-2 and Tektin-2 transcripts were first expressed in the meiotic germ cells (pachytene spermatocytes) and their expression was upregulated in the post-meiotic germ cells (round spermatids). Sequential extraction of sperm proteins showed that, Tektin-2 was majorly extracted out in the Triton X-100 and DTT fraction, whereas, ODF-2 was maximally extracted in the presence of urea and DTT.

In conclusion, these observations indicate that IGF1R and Src family of tyrosine kinases are critical for mammalian sperm capacitation and associated global PYP. Inhibition of sperm capacitation was associated with hypo-tyrosine-phospohorylation of certain proteins associated with mitochondrial membrane, axonemal structures and outer dense fibers of the sperm flagellum. Future work can be directed towards understanding the role of other RTKs and NRTKs involved in sperm capacitation and the molecular characterization of hypophosphorylated proteins critical for sperm function and its fertilization competence.